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Analysis of mutant NS5B proteins encoded by isolates from chimpanzees chronically infected following clonal HCV RNA inoculation

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Abstract

We hypothesized that mutations in the HCV NS5B polymerase, which occur during infection, may affect RNA-dependent RNA polymerase (RdRp) activity. NS5B proteins corresponding to a genotype 1a infectious clone and mutants identified in chimpanzees following inoculation with the clone were expressed and purified and their in vitro RdRp activity was compared to a NS5B genotype 1b control. A Gln-65-to-His mutation increased RdRp activity by 1.8-fold as compared to the infectious clone. Moreover, this NS5B1a protein had RdRp activity similar to the NS5B1b control. Three NS5B proteins representing mutations found in another animal had no in vitro RdRp activity. All mutations were maintained in the majority circulating virus for at least 216 weeks. The results demonstrate that some in vivo mutations of NS5B directly enhance in vitro RdRp activity. In addition, they suggest that the in vitro RdRp activity of NS5B may not always reflect in vivo activity within replication complexes.

Keywords: Hepatitis C virus; Plus-strand RNA viruses; RNA replication; Nonstructural protein 5B; NS5B; RNA-dependent RNA polymerase

Introduction

HCV is a positive-sense single-stranded RNA virus with a genome of approximately 9600 nucleotides that chronically infects at least 4 million people in the USA (Choo et al., 1991; Thomas, 2000). The genome has a conserved 5′UTR with an internal ribosome entry site (IRES) for translation of a polyprotein consisting of approximately 3011 amino acids (Han et al., 1991; Rijnbrand and Lemon, 2000; Kong and Sarnow, 2002; Otto et al., 2002). A conserved genomic 3′UTR stem loop is essential for virus replication in vivo and is probably involved in the initiation of negative-strand synthesis (Kolykhalov et al., 2000; Blight and Rice, 1997; Yanagi at al., 1999; Friebe and Bartenschlager, 2002). The viral polyprotein is processed co- and posttranslationally into the individual structural and nonstructural proteins. The NS5B protein encodes an RNA-dependent RNA polymerase (RdRp), which has a structure consisting of fingers, palm, and thumb domains (Ago et al., 1999; Bressanelli et al., 1999; Lesburg et al., 1999). The lambda 1 loop of HCV NS5B, which extends from the fingers to the thumb domain, is unique to RNA-dependent RNA polymerases and the beta-hairpin toward the catalytic region is specific for HCV NS5B. In addition to these characteristics, HCV NS5B has a number of enzymatic and structural differences from cellular RNA polymerases which make it a promising target for small molecule antivirals.

The cellular and molecular mechanisms favoring HCV persistence remain undefined. Although many of them are likely to involve viral–host interactions which affect inter-

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feron-regulated antiviral pathways or the immune system (Foy et al., 2003), mutations in the viral genome that affect RNA replication may also be involved. This may include the NS5B polymerase, 3′ or 5′ structures of the (+) or (−) strand RNA, or other viral nonstructural proteins that form components of the replication complex.

Due to the lack of a small animal model or an efficient cell-culture system for HCV, much remains to be learned about the molecular mechanisms of HCV RNA replication. Nevertheless, recombinant NS5B purified from insect cells infected with recombinant baculovirus or *Escherichia coli* expressed recombinant protein have been used to define several basic characteristics of the HCV RdRp (Behrens et al., 1996; Lohmann et al., 1997; Al et al., 1998). The properties that have been defined include the effects of pH, salt, and a requirement for either Mg^{2+} or Mn^{2+} (Lohmann et al., 1998; Ferrari et al., 1999). HCV NS5B can support either primer-dependent or primer-independent synthesis of RNA from an HCV template by de novo initiation if the extreme 98-nt 3′-end stem loop of genomic RNA is present (Lohmann et al., 1998; Zhong et al., 2000; Kao et al., 2000; Oh et al., 2000; Ranjith-Kumar et al., 2002). GTP stimulates in vitro NS5B polymerase activity by 50- to 100-fold through a mechanism that most likely involves a rate-limiting step of initiation (Lohmann et al., 1999). The recent identification of a GTP binding site on the surface of the NS5B apoprotein that is distant from the catalytic site raises the possibility that the enzyme might be regulated allosterically (Bressanelli et al., 2002).

Few studies have examined the in vivo sequence changes that occur in the NS5B coding region during chronic HCV infection. The quasispecies nature of isolates from patients complicates such studies. However, chimpanzees chronically infected following intrahepatic inoculation with clonal genotype 1a HCV RNA have a single starting sequence which facilitates the identification of subsequent mutations which are maintained in the dominant viral genome (Kolykhalov et al., 1997; Major and Feinstone, 2000). Two animals (Ch1535 and Ch1536) which became chronically infected following inoculation with in vitro transcribed HCV RNA have been followed over several years and the dominant virus sequence was determined at 22, 26, 60, 130, and 216 weeks postinoculation (p.i.) (Major et al., 1999). Direct sequencing was carried out on overlapping PCR products covering 100% of the coding region and parts of the 5′ and 3′ UTRs. This method of analysis was chosen because it accurately determines the sequence of the dominant circulating virus.

Four amino acid mutations in the NS5B region were observed during the first 60 weeks of infection, one in Ch1535 and three in Ch1536 (Major et al., 1999). A Gln-65-to-His mutation in the fingers domain appeared after 60 weeks of infection in Ch1535, a His-33-to-Asn mutation in the lambda 1 loop appeared at 22 weeks, and both a Leu-36-to-Met mutation in the lambda 1 loop and Asp-135-to-Asn in the fingers domain appeared after 60 weeks of infection in Ch1536. All of these mutations were stably maintained in the dominant circulating virus throughout the follow-up analysis period (216 weeks p.i.).

In this study, we report the construction of NS5B clones that mimic the natural mutations observed in chimpanzees chronically infected with clonal genotype 1a HCV RNA and the in vitro analysis of RdRp activity of these mutants and control NS5B proteins. These studies provide evidence that some mutations occurring in the NS5B region during chronic infection may result in a major increase in viral RNA replication. However, our results also indicate that the in vitro RdRp assays of purified NS5B proteins may not always reflect their in vivo activity within replication complexes.

Results and discussion

Amino acid sequence comparison of selected NS5B genotype 1a and 1b proteins

The amino acid sequence of NS5B encoded by the genotype 1a infectious clone (H77; AF009606) was aligned with two genotype 1b NS5B proteins demonstrated to have in vitro RdRp activity (Fig. 1). The genotype 1b NS5B protein used as a control was similar to a previously reported genotype 1b NS5B protein (AJ238799 con1/1b) (Fig. 1) (Lohmann et al., 1997). Consistent with previously described differences between other genotype 1a and 1b subtypes, the 1a and 1b NS5B proteins used in this study were different at 61 amino acid residues (10.7%) (Fig. 1) (Hagedorn et al., 2000).

Expression and purification of NS5B1a proteins encoded by an infectious clone and mutants occurring during chronic infection

Vectors to express the NS5B protein encoded by the genotype 1a infectious clone (designated wild-type) and mutants appearing during infection were prepared as described under Materials and methods. The Gln-65-to-His mutation (M1) occurred between 26 and 60 weeks of infection in Ch1535 (Table 1)(Major et al., 1999). In Ch1536 the His-33-to-Asn mutation (M2) was observed at 22 weeks and the Leu-36-to-Met plus the Asp-135-to-Asn mutations (M3) were observed at 60 weeks of infection. At the end of 60 weeks of infection the majority circulating virus in Ch 1536 contained all three of these mutations (M4) (Table 1). All mutations in both animals were maintained as the majority circulating virus, as determined by direct sequencing of PCR products, for at least 216 weeks (>4 years postinoculation), indicating that they were stable mutations which had

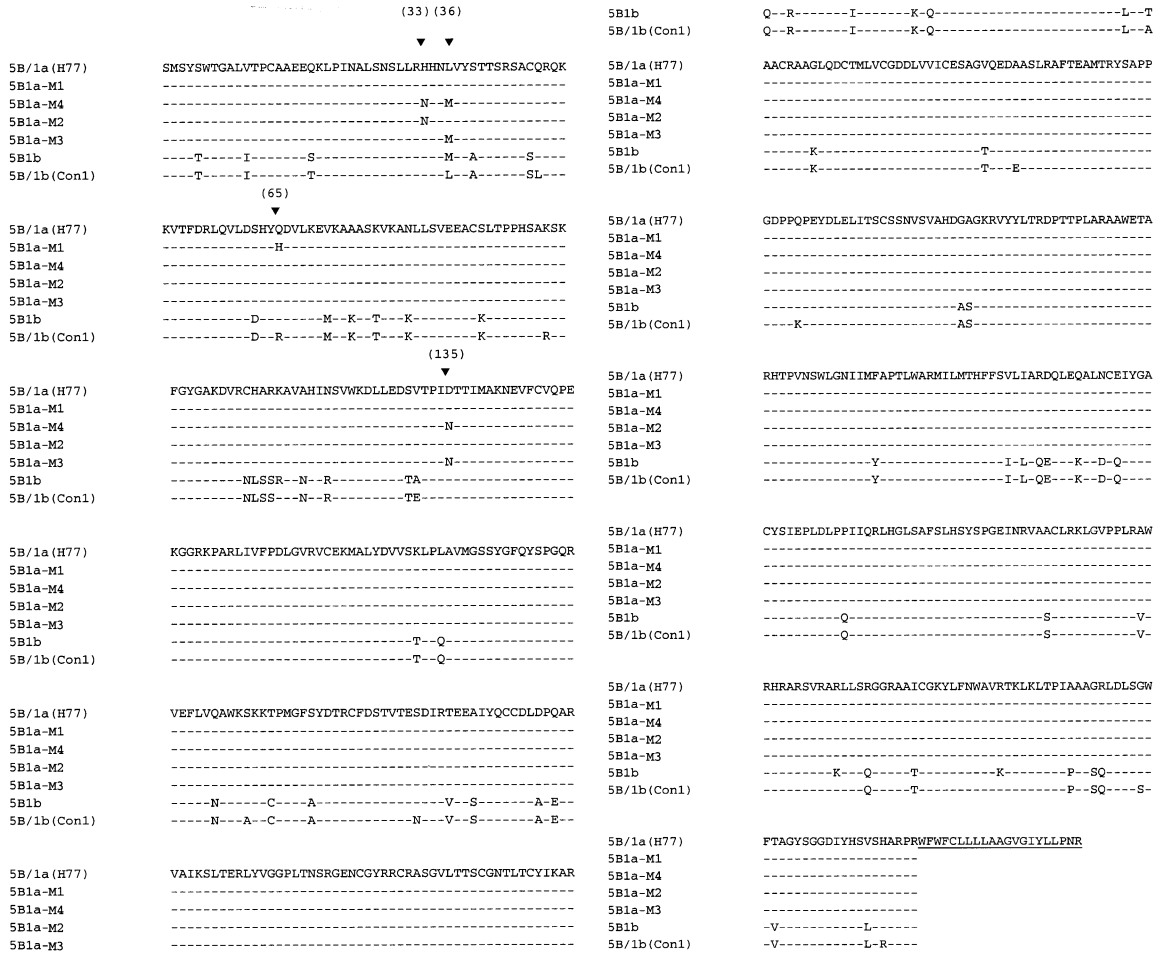

Fig. 1. Sequence alignments are shown for the HCV NS5B polymerase encoded by the genotype la infectious clone (H77, AF009606), the four mutants of NS5B1a that were identified in chronically infected chimpanzees (M1–M4), the NS5B genotype 1b control protein (5B1b), and a previously reported NS5B1b protein (Con1, AJ238799). Arrowheads indicate the position of mutations and their location in the recombinant protein. The 21 amino acids at the C-terminus (underlined) were not present in the recombinant protein and were replaced by a hexahistidine tag of the pET-21a vector to increase protein yields and ease of purification. Note that the purified recombinant protein has an additional SM at the amino-terminus as compared to the naturally occurring HCV NS5B protein that is produced by proteolytic processing of the polyprotein (Al et al., 1998).

been incorporated into the primary replicating HCV genome (Major et al., 1999; Major and Feinstone, 2000). To further verify that these mutations were incorporated into the majority circulating HCV genome at 216 weeks postinoculation, multiple clones of PCR fragments covering the NS5B region from each animal were isolated, purified, and sequenced. In Ch1535, eight of nine clones carried the Gln-65-to-His mutation (M1), while only one had the wild-type sequence. In Ch1536, 9/10 clones carried the mutations at residues 33 and 36 and 1/10 represented the wild-type sequence, while 9/10 clones carried the mutation at residue 135 and 1/10 represented the wild-type sequence.

Wild-type and mutant NS5B proteins were expressed in *E. coli* and purified as described under Materials and methods (Al et al., 1998). Approximately 3 to 4 mg of greater than 90% pure recombinant NS5B was recovered per liter of culture for all proteins (Table 2 and Fig. 2).

RdRp activity of the NS5B proteins encoded by an infectious clone and in vivo mutants

To compare the activity of NS5B encoded by the HCV genotype 1a infectious clone with the mutants of NS5B that developed during chronic infection, RdRp assays were done with the respective recombinant NS5B proteins and RNA templates representing the 3′end of HCV genomic RNA. The HCV NS5B polymerase can use a variety of RNA templates and includes the 3′X-tail of HCV RNA which permits primer independent de novo RNA synthesis (Zhong et al., 2000; Luo et al., 2000; Ranjith-Kumar et al., 2002). We used 3′X(+) HCV RNA as template in the RdRp assays. The relative RdRp activity of NS5B encoded by the 1a infectious clone was 74% of the NS5B1b control (Fig. 3). This was consistent with previous reports suggesting that NS5B proteins from genotype 1a viruses have less RdRp activity than genotype 1b viruses (Ferrari et al., 1999;

Table 1
Mutations of NS5B1a following infection with clonal HCV RNA

Name	Animal	Amino acid position[a]	Amino acid change	Nucleotide change	Mutation appeared time (weeks)
NS5B1a-M1	Ch.1535	65	Gln-His	CAG-CAT	26–60
NS5B1a-M2	Ch.1536	33	His-Asn	CAT-AAT	4–22
NS5B1a-M3	Ch.1536	36	Leu-Met	CTG-ATG	22–60
		135	Asp-Asn	GAC-AAC	22–60
NS5B1a-M4	Ch.1536	33	His-Asn	CAT-AAT	4–22
		36	Leu-Met	CTG-ATG	22–60
		135	Asp-Asn	GAC-AAC	22–60

[a] See Fig. 1.

Friebe et al., 2002). However, the mutation of Gln-65 of NS5B, a hydrophilic polar amino acid located on the surface of the fingers domain, to the hydrophilic basic amino acid His (M1) resulted in a 1.8-fold increase (P value by t test <0.0001) in RdRp activity relative to NS5B of the infectious clone (Figs. 3 and 4). Moreover, this resulted in a genotype 1a NS5B protein with slightly more RdRp activity than the NS5B1b control (Fig. 3). This result provides an example where a NS5B polymerase encoded by a genotype 1a virus does have in vitro RdRp activity that is at least equal to a genotype 1b NS5B polymerase and may be useful in modifying genotype 1a HCV replicons (Blight et al., 2003).

Previous efforts to identify point mutations that increase the RdRp activity of a genotype 1a NS5B protein, based on sequence analysis of 48 viral isolates and altering a specific isolate toward a consensus sequence, did not result in major increases in activity (Hagedorn et al., 2000; Hagedorn et al., unpublished data). The precise reason why the Gln-to-His mutation at position 65 enhanced in vitro RdRp activity by as much as 1.8-fold remains unknown at this time. Nevertheless, the location of Gln-65 in the NS5B crystal structure is close to the Arg-345 residue which stimulates RdRp activity two- to threefold when mutated to Lys (Fig. 4) (Lohmann et al., 1998). It remains to be determined if these two mutations share a similar mechanism or have independent mechanisms which either modify properties of the proteins in solution or directly increase RdRp catalytic activity.

The mutations of NS5B that occurred in Ch1536 and were maintained in the dominant circulating virus did not stimulate in vitro RdRp activity. In fact, the NS5B1a-M2 (His-33 to Asn), M3 (Leu-36 to Met plus the Asp-135 to Asn), and M4 (all three mutations) proteins had essentially no detectable in vitro RdRp activity (Figs. 3 and 4). This result remains a puzzle and several possible explanations can be considered. His-33 is part of the lambda 1 loop of HCV NS5B, which includes amino acids 11–45 within the fingers domain and is structurally unique among RNA-dependent RNA polymerases (Bressanelli et al., 1999; Lesburg et al., 1999; Ago et al., 1999). This loop forms two α-helical turns which pack closely against helices of the thumb subdomain (Fig. 5). The lambda 1 loop has been suggested to participate in the concerted "clamping" motion of the fingers and thumb domains during the translocation of a template along the polymerase. His-33 hydrogen bonds to residue 493 at the C-terminus of helix S in the thumb

Table 2
Purification table: recombinant NS5B1a expressed in *E. coli*

	Volume (ml)	Protein concentration (mg/ml)	Total protein (mg)
E. coli culture	1000		
Lysate	80	30.5	2440
HPLC affinity column	24	0.16	3.5

Note. The data in the table were obtained with the NS5B protein encoded by the infectious clone (wt) and is representative of the purification of other recombinant NS5B proteins (M1–M4 and the genotype 1b control). The yield of *E. coli* cell pellets was 4 g wet weight per 1000 ml of culture and "Lysate" represents the 40,000 *g* supernatant following sonication of cells in lysis buffer. Details of the purification method are provided under Materials and methods.

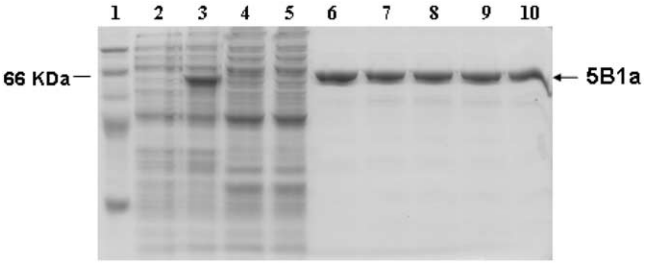

Fig. 2. Expression in *E. coli* and purification of recombinant HCV NS5B1a encoded by the infectious clone (wild-type) and mutants that occurred during chronic infection. The expression and purification of proteins are described under Materials and methods. Representative stages of expression and purification were analyzed by 10% SDS–PAGE and the proteins were stained with Coomassie blue. The gel shown represents: lane 1, molecular mass standards (97, 66, 45, 35, and 29 kDa); lane 2, uninduced *E. coli* lysate; lane 3, IPTG-induced *E. coli* lysate; lane 4, starting material prepared from IPTG-induced *E. coli* cells; lane 5, a representative flowthrough of purifications; lanes 6–10, purified recombinant NS5B proteins encoded by the infectious clone (lane 6) and mutants that appeared during chronic infection in two chimpanzees (M1–M4 in lanes 7–10, respectively; see Table 1). The NS5B1b control protein was purified by the same method and was of similar purity (not shown).

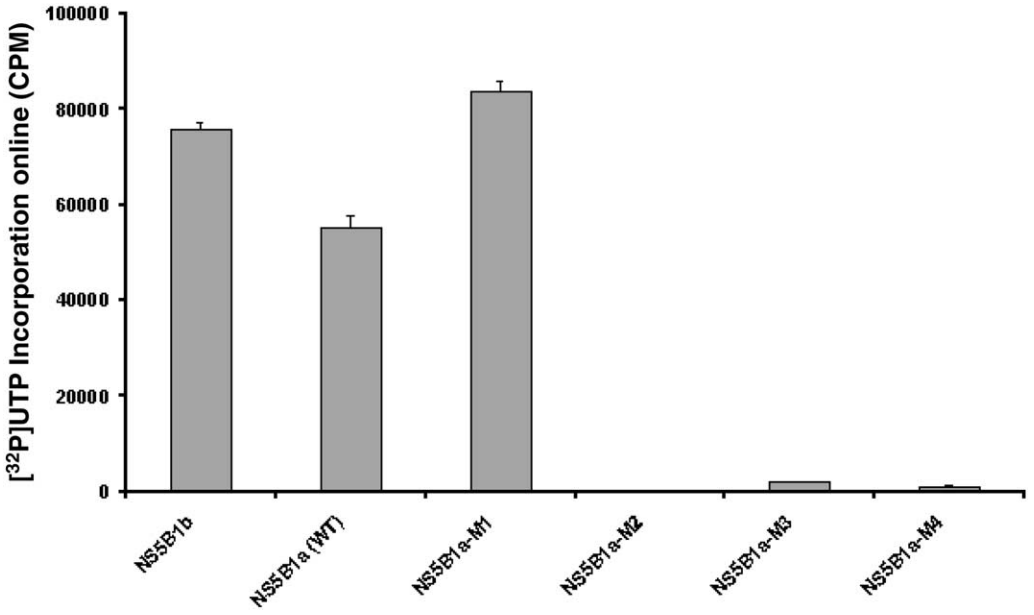

Fig. 3. RdRp activity of recombinant NS5B1a polymerase encoded by an infectious clone and mutants that occurred during chronic infection as compared to a NS5B1b control protein. The synthesis of [^{32}P]UTP-labeled RNA (mean ± SEM; $n = 6$) from a HCV 3′X (+) template is shown in the bar graph. RdRp assays were as described under Materials and methods.

domain and may be a critical element in the interaction between the fingers and thumb domains (Bressanelli et al., 1999). In addition, this site of interaction is also close to the GTP binding pocket that has been proposed to regulate NS5B allosterically (Bressanelli et al., 2002). Leu-36, within the lambda 1 loop, also participates in the fingers–thumb interaction. Previous studies have also shown that deletion of the first 40 residues of NS5B resulted in complete loss of RdRp activity (Lohmann et al., 1998). In

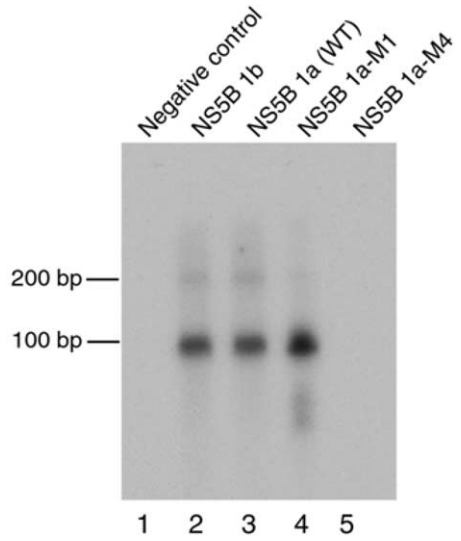

Fig. 4. Gel analysis of RNA products. RNA products from RdRp assays using HCV 3′X(+) RNA as template (98 nt in length), incubated at 30°C for 1 h, were analyzed by 5% polyacrylamide–7 M urea gel electrophoresis and autoradiography (see Materials and methods). The autoradiogram from a representative experiment (done three times) is shown: lane 1, control incubation with no NS5B added; and complete incubations with the specified NS5B protein (lanes 2 to 5; as described under-Materials and methods). The locations of RNA markers (Novagen) are shown.

Fig. 5. Location of the mutations in the HCV NS5B polymerase. A ribbons diagram of NS5B (finger, palm, and thumb domains colored red, yellow, and blue, respectively) is shown depicting the location and side chains of the His-33, Leu-36, Gln-65, and Asp-135 residues that were mutated and studied. Note that the location of Gln-65, which stimulated RdRp activity when mutated to His, is close to another site (Arg-345), which stimulates activity when mutated to Lys (Lohmann et al., 1998).

addition, both Glu-18 and Leu-30 within the lambda 1 loop have been reported to be critical for in vitro RdRp activity (Labonte et al., 2002; Qin et al., 2002). However, all of the NS5B mutations observed in Ch1536 were present in the majority circulating virus and were stably maintained as the consensus sequence for at least 4 years postinfection, suggesting the resulting NS5B protein is expressed by replicating viruses and has sufficient in vivo RdRp activity to maintain a chronic infection. We suggest that recombinant NS5B in solution may be particularly sensitive to changes in residues that participate in the fingers–thumb interactions and that when NS5B is anchored within a replication complex in vivo the specific mutations discussed do not disrupt RdRp activity to the same degree. On the other hand, changes at the M2–4 sites may modify NS5B:NS5B interactions and oligomerization (Table 1) (Wang et al., 2002). Another possible explanation is that mutations identified in the NS3 and NS5A proteins, which are predicted to be present in replication complexes, are compensatory and attenuate the in vivo effect of the mutations at residues 33, 36, and 135 of NS5B (Major et al., 1999). We are in the process of further testing the effect of these mutations on HCV RNA synthesis in a cell-based replicon system.

The events that control clearance of HCV or progression to persistent infection have not been identified. There are indications that stronger and broader T cell responses during the acute phase of infection correlate with clearance. However, functional studies of mutations identified during chronic infection using clinical isolates are complicated by the presence of quasispecies and the inability to distinguish nonfunctional viral sequences from those contributing to replication. On the other hand, inoculation of chimpanzees with cloned HCV RNA permits a clear identification of mutations that occur during chronic infection. Our results demonstrate that some mutations which develop in the NS5B coding region during chronic HCV infection may have major implications for the efficiency of HCV RNA replication and viral persistence.

Stable amino acid mutations observed in other regions of serum-derived HCV RNA from Ch1535 and Ch1536 have been identified and are likely to include other components of the replication complex. Mutations in NS3 (A1533P and V1635I) identified at week 26 in Ch1535 and NS3 (R1181K) arising at week 22 and NS5A (T2349A and A2412T) identified at week 60 in Ch1536 have been described (Major et al., 1999). These changes may represent adaptive mutations that complement those identified at residue 33, 36, and 135 in NS5B to achieve effective RNA replication. Complementary mutations have been identified in the NS3 and NS5A regions during cell culture of HCV subgenomic replicons (Blight et al., 2000; Krieger et al., 2001). In addition, several of the mutations observed in Ch1535 and Ch1536 during the early phase of infection could confer CTL escape (Major and Feinstone, 2000). Adaptive mutations in other proteins involved in the replication complex might be needed to maintain virus survival. The NS3 residue at position 1635 has been previously identified as part of a cytotoxic lymphocyte (CTL) epitope in chimpanzees, although the V1635I change has not been confirmed as a CTL escape mutant in Ch1535 (Cooper et al., 1999). Although the in vivo biological role for the stable mutations in the NS5B region observed during chronic HCV infection of chimpanzees remains to be determined, the ability of at least one of them to produce major changes of in vitro RdRp activity may have practical implications for the design of small molecule therapeutics targeting the NS5B polymerase (De Francesco et al., 2003; Gu et al., 2003; Dhanak et al., 2002). Our results suggest that further studies of NS5B mutations which occur in model systems or patients with chronic hepatitis C are warranted.

Materials and methods

Construction of expression plasmids

The pET-21a vector (Novagen, Madison, WI) was used to express recombinant NS5B with a C-terminus hexahistidine tag in *E. coli* following previously described methods (Al et al., 1998). A full-length NS5B cDNA of HCV genotype 1a corresponding to the sequence of the original infectious cDNA clone used to inoculate the chimpanzees was cloned into pCR2.1 (Invitrogen) and used as a template in these studies (Kolykhalov et al., 1997; Major et al., 1999; Major and Feinstone, 2000). The NS5B-region was amplified by PCR using the following primers: 5′-TTATTGGATCCA TGGCTAGCATGTCTTATTCCTGGACAGGCGC-3′ (forward primer), 5′-AACATCTCGAGGCGGGGGC CGGGCATGAGACACG-3′ (reverse primer). The underlined sequences represent the *Nhe*I and *Xho*I subcloning sites, respectively. The final PCR product was cloned into pET-21a downstream of the T7 RNA polymerase promoter using the *Nhe*I and *Xho*I sites. The C-terminal 21 amino acids of NS5B were deleted to produce a more soluble recombinant protein (Ferrari et al., 1999). This construct was used as a template to introduce the point mutations into HCV NS5B1a observed in the chronically infected chimpanzees. NS5B1a with substitution of Gln-65 with His was designated M1; substitutions of His-33 with Asn, Leu-36 with Met, and Asp-135 with Asn were designated M4; substitution of His-33 with Asn was designated M2; and substitutions of Leu 36 with Met and Asp-135 with Asn were designated M3 (Table 1 and Fig. 1). These mutations were produced by site-directed mutagenesis and were confirmed by sequencing as previously described (Spivak-Kroizman et al., 2002). An NS5B genotype 1b expression vector was used as a control. The amino acid sequences of the NS5B isolates were aligned using CLUSTAL W software (Thompson et al., 1994).

Expression and purification of the recombinant NS5B protein

NS5B1a (infectious clone), M1, M2, M3, M4, and NS5B1b proteins were expressed in *E. coli* strain BL21 (DE3) following previously described methods (Al et al., 1998). BL21 cells were grown to an OD_{600} of 0.5 at 37°C in TB medium containing carbenicillin and protein expression was induced at 25°C for 6 h with 1 mM IPTG. Cell pellets were suspended in lysis buffer (50 mM HEPES, 400 mM NaCl, 10% glycerol, 0.1% NP-40, 1 mM PMSF, 10 μg/ml each of aprotinin, leupeptin, pepstatin A, and 0.1 mM benzamidine; pH 8.0) and sonicated or lysed using a French Press and centrifuged for 30 min at 40,000 *g*. The supernatant was applied to a FPLC TALON Superflow Metal Affinity Column (Clontech). The resin was washed and the recombinant proteins were eluted with an imidazole gradient (1 to 500 mM). The eluted protein was dialyzed against 50 mM HEPES, 300 mM NaCl, 10% glycerol, 0.1% NP-40, 1 mM DTT (pH 8.0) and stored at −80°C. Proteins were analyzed by 10% SDS–PAGE and quantitated using a Bio-Rad protein assay.

Preparation of RNA templates

To prepare HCV RNA templates, a full-length serum-derived clone of HCV in pGEM-T (Promega) was used. The HCV 3′ 98-nt stem loop of genomic HCV RNA designated 3′X (+) was used in these studies. The following primer sets were used to amplify PCR products for T7 RNA polymerase synthesis of RNA templates: forward primer 5′-AGTTAATACGACTCACTATAGGGAGGTGGCTCC-3′ and reverse primer 5′-GAGTACTTGATCTGCAGAGAGGC-3′ for 3′X (+). The T7 RNA polymerase promoter sequence is underlined. The RNA templates were synthesized with T7 polymerase using conditions suggested by the manufacturer (Epicentre Technologies). The RNA transcripts were treated with DNase I, phenol-chloroform extracted, and ethanol precipitated. RNA was analyzed by 1% agarose gel electrophoresis.

RdRp assay

RdRp assays were performed in 50-μl incubations containing 50 mM HEPES (pH 8.0), 5 mM $MnCl_2$, 4 mM DTT, 0.5 mM each ATP, CTP, and GTP, 5 μCi of [α-^{32}P]UTP (Amersham Biosciences), 200 ng of RNA template, and 500 ng of the purified NS5B as described elsewhere (Al et al., 1998). The reaction mixtures were incubated at 30°C for 1 h. The RNA synthesized was precipitated by adding 10 μg of calf thymus DNA plus 7% trichloroacetic acid (TCA) and incubated on ice for 30 min. The synthesized RNA was collected by using GF/C glass filters (Whatman) and washed with pyrophosphate followed by 95% ethanol. The amount of radioactivity bound to each filter was determined by liquid scintillation counting. RNA products were also analyzed by electrophoresis using a 5% denaturing polyacrylamide–7 M urea gel and autoradiography.

Cloning and sequencing of PCR product

PCR products covering the NS5B region containing the observed mutations were generated as previously described (Major et al., 1999). The PCR fragments were cloned into the pCR2.1 TA cloning vector (Invitrogen, Carlsbad, CA) according to the instructions of the manufacturer. Positive clones were determined by restriction enzyme digestion with *Eco*RI and sequenced using the PE Applied Biosystems Dye Terminator Cycle Sequencing FS reaction mix according to the instructions of the manufacturer (PE Applied Biosystems, Foster City, CA). Sequences were compared using DNAStar Seqman software (DNAStar Inc., Madison, WI).

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References

Ago, H., Adachi, T., Yoshida, A., Yamamoto, M., Habuka, N., Yatsunami, K., Miyano, M., 1999. Crystal structure of the RNA-dependent RNA polymerase of hepatitis C virus. Structure 7, 1417–1426.

Al, R.H., Xie, Y., Wang, Y., Hagedorn, C.H., 1998. Expression of recombinant hepatitis C virus non-structural protein 5B in Escherichia coli. Viral Res. 53, 141–149.

Behrens, S.E., Tomei, L., De Francesco, R., 1996. Identification and properties of the RNA-dependent RNA polymerase of hepatitis C virus. EMBO J. 15, 12–22.

Blight, K.J., Rice, C.M., 1997. Secondary structure determination of the conserved 98-base sequence at the 3′ terminus of hepatitis C virus genome RNA. J. Virol. 71, 7345–7352.

Blight, K.J., Kolykhalov, A.A., Rice, C.M., 2000. Efficient initiation of HCV RNA replication in cell culture. Science 290, 1972–1974.

Blight, K.J., McKeating, J.A., Marcotrigiano, J., Rice, C.M., 2003. Efficient replication of hepatitis C virus genotype 1a RNAs in cell culture. J. Virol. 77, 3181–3190.

Bressanelli, S., Tomei, L., Roussel, R., Incitti, I., Vitale, R.L., Mathieu, M., Francesco, R.D., Rey, F.A., 1999. Crystal structure of the RNA-dependent RNA polymerase of hepatitis C virus. Proc. Natl. Acad. Sci. USA 96, 13034–13043.

Bressanelli, S., Tomei, L., Rey, F.A., Francesco, R.D., 2002. Structural analysis of the hepatitis C virus RNA polymerase in complex with ribonucleotides. J. Virol. 76, 3482–3492.

Choo, Q.L., Richman, K.H., Han, J.H., Berger, K., Lee, C., Dong, C., Gallegos, C., Coit, D., Medina-Selby, R., Barr, P.J., Weiner, A.J., Bradley, D.W., Kuo, G., Houghton, M., 1991. Genetic organization and

diversity of the hepatitis C virus. Proc. Natl. Acad. Sci. USA 88, 2451–2455.

Cooper, S., Erickson, A.L., Adams, E.J., Kansopon, J., Weiner, A.J., Chien, D.Y., Houghton, M., Parham, P., Walker, C.M., 1999. Analysis of a successful immune response against hepatitis C virus. Immunity 10, 439–449.

De Francesco, R., Tomei, L., Altamura, S., Summa, V., Migliaccio, G., 2003. Approaching a new era for hepatitis C virus therapy: inhibitors of the NS3-4A serine protease and the NS5B RNA-dependent RNA polymerase. Antiviral Res. 58, 1–16.

Dhanak, D., Duffy, K.J., Johnston, V.K., Lin-Goerke, J., Darcy, M., Shaw, A.N., Gu, B., Silverman, C., Gates, A.T., Nonnemacher, M.R., Earnshaw, D.L., Casper, D.J., Kaura, A., Baker, A., Greenwood, C., Gutshall, L.L., Maley, D., Del Vecchio, A., Macarron, R., Hofmann, G.A., Alnoah, Z., Cheng, H.Y., Chan, G., Khandekar, S., Keenan, R.M., Sarisky, R.T., 2002. Identification and biological characterization of heterocyclic inhibitors of the hepatitis C virus RNA-dependent RNA polymerase. J. Biol. Chem. 277, 38322–38327.

Ferrari, E., Wright-Minogue, J., Fang, J.W., Baroudy, B.M., Lau, J.N., Hong, Z., 1999. Characterization of soluble hepatitis C virus RNA-dependent RNA polymerase expressed in Escherichia coli. J. Virol. 73, 1649–1654.

Foy, E., Li, K., Wang, C., Sumpter Jr., R., Ikeda, M., Lemon, S.M., Gale Jr., M., 2003. Regulation of interferon regulatory factor-3 by the hepatitis C virus serine protease. Science 300, 1145–1148.

Friebe, P., Lohmann, V., Krieger, N., Bartenschlager, R., 2001. Sequences in the 5′ nontranslated region of hepatitis C virus required for RNA replication. J. Virol. 75, 12047–12057.

Friebe, P., Bartenschlager, R., 2002. Genetic analysis of sequences in the 3′ nontranslated region of hepatitis C virus that are important for RNA replication. J. Virol. 76, 5326–5338.

Gu, B., Johnston, V.K., Gutshall, L.L., Nguyen, T.T., Gontarek, R.R., Darcy, M.G., Tedesco, R., Dhanak, D., Duffy, K.J., Kao, C.C., Sarisky, R.T., 2003. Arresting initiation of hepatitis C virus RNA synthesis using heterocyclic derivatives. J. Biol. Chem. 278, 16602–16607.

Hagedorn, C.H., Van Beers, E.H., De Staercke, C., 2000. Hepatitis C virus RNA-dependent RNA polymerase (NS5B polymerase). Curr. Top. Microbiol. Immunol. 242, 225–260.

Han, J.H., Shyamala, V., Richman, K.H., Brauer, M.J., Irvine, B., Urdea, M.S., Tekamp-Olson, P., Kuo, G., Choo, Q.L., Houghton, M., 1991. Characterization of the terminal regions of hepatitis C viral RNA: identification of conserved sequences in the 5′ untranslated region and poly (A) tails at the 3′ end. Proc. Natl. Acad. Sci. USA 88, 1711–1715.

Kao, C.C., Yang, X., Kline, A., Wang, Q.M., Barket, D., Heinz, B.A., 2000. Template requirements for RNA synthesis by a recombinant hepatitis C virus RNA-dependent RNA polymerase. J. Virol. 74, 11121–11128.

Kolykhalov, A.A., Agapov, E.V., Blight, K.J., Mihalik, K., Feinstone, S.M., Rice, C.M., 1997. Transmission of hepatitis C by intrahepatic inoculation with transcribed RNA. Science 277, 570–574.

Kolykhalov, A.A., Mihalik, K., Feinstone, S.M., Rice, C.M., 2000. Hepatitis C virus-encoded enzymatic activities and conserved RNA elements in the 3′ nontranslated region are essential for virus replication in vivo. J. Virol. 74, 2046–2051.

Kong, L.K., Sarnow, P., 2002. Cytoplasmic expression of mRNAs containing the internal ribosome entry site and 3′ noncoding region of hepatitis C virus: effects of the 3′ leader on mRNA translation and mRNA stability. J. Virol. 76, 12457–12462.

Krieger, N., Lohmann, V., Bartenschlager, R., 2001. Enhancement of hepatitis C virus RNA replication by cell culture-adaptive mutations. J. Virol. 75, 4614–4624.

Labonte, P., Axelrod, V., Agarwal, A., Aulabaugh, A., Amin, A., Mak, P., 2002. Modulation of hepatitis C virus RNA-dependent RNA polymerase activity by structure-based site-directed mutagenesis. J. Biol. Chem. 277, 38838–38846.

Lesburg, C.A., Cable, M.B., Ferrari, E., Hong, Z., Mannarino, A.F., Weber, P.C., 1999. Crystal structure of the RNA-dependent RNA polymerase from hepatitis C virus reveals a fully encircled active site. Nature Struct. Biol. 6, 937–943.

Lohmann, V., Korner, F., Herian, U., Bartenschlager, R., 1997. Biochemical properties of hepatitis C virus NS5B RNA-dependent RNA polymerase and identification of amino acid sequence motifs essential for enzymatic activity. J. Virol. 71, 8416–8428.

Lohmann, V., Roos, A., Korner, F., Koch, J.O., Bartenschlager, R., 1998. Biochemical and kinetic analyses of NS5B RNA-dependent RNA polymerase of the hepatitis C virus. Virology 249, 108–118.

Lohmann, V., Overton, H., Bartenschlager, R., 1999. Selective stimulation of hepatitis C virus and pestivirus NS5B RNA polymerase activity by GTP. J. Biol. Chem. 274, 10807–10815.

Luo, G., Hamatake, R.K., Mathis, D.M., Racela, J., Rigat, K.L., Lemm, J., Colonno, R., 2000. De novo initiation of RNA synthesis by the RNA-dependent RNA polymerase (NS5B) of hepatitis C virus. J. Virol. 74, 851–863.

Major, M.E., Feinstone, S.M., 2000. Characterization of hepatitis C virus infectious clones in chimpanzees: long-term studies. Curr. Top. Microbiol. Immunol. 242, 279–298.

Major, M.E., Mihalik, K., Fernandez, J., Seidman, J., Kleiner, D., Kolykhalov, A.A., Rice, C.M., Feinstone, S.M., 1999. Long-term follow-up of chimpanzees inoculated with the first infectious clone for hepatitis C virus. J. Virol. 73, 3317–3325.

Oh, J.W., Sheu, G.T., Lai, M.M., 2000. Template requirement and initiation site selection by hepatitis C virus polymerase on a minimal viral RNA template. J. Biol. Chem. 275, 17710–17717.

Otto, G.A., Lukavsky, P.J., Lancaster, A.M., Sarnow, P., Puglisi, J.D., 2002. Ribosomal proteins mediate the hepatitis C virus IRES-HeLa 40S interaction. RNA 8, 913–923.

Spivak-Kroizman, T., Friedland, D.E., De Staercke, C., Gernert, K.M., Goss, D.J., Hagedorn, C.H., 2002. Mutations in the S4-H2 loop of eIF4E which increase the affinity for m7GTP. FEBS Lett. 516, 9–14.

Qin, W., Luo, H., Nomura, T., Hayashi, N., Yamashita, T., Murakami, S., 2002. Oligomeric interaction of hepatitis C virus NS5B is critical for catalytic activity of RNA-dependent RNA polymerase. J. Biol. Chem. 277, 2132–2137.

Ranjith-Kumar, C.T., Gutshall, L., Kim, M.J., Sarisky, R.T., Kao, C.C., 2002. Requirements for de novo initiation of RNA synthesis by recombinant flaviviral RNA-dependent RNA polymerases. J. Virol. 76, 12526–12536.

Rijnbrand, R.C., Lemon, S.M., 2000. Internal ribosome entry site-mediated translation in hepatitis C virus replication. Curr. Top. Microbiol. Immunol. 242, 85–116.

Thomas, D.L., 2000. Hepatitis C epidemiology. Curr. Top. Microbiol. Immunol. 242, 25–41.

Thompson, J.D., Higgins, D.G., Gibson, T.J., 1994. CLUSTAL W. Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. 22, 4673–4680.

Wang, Q.W., Hockman, M.A., Staschke, K., Johnson, R.B., Case, K.A., Lu, J., Parsons, S., Zhang, F., Rathnachalam, R., Kirkegaard, K., Colacino, J.M., 2002. Oligomerization and cooperative RNA synthesis activity of hepatitis C virus RNA-dependent RNA polymerase. J. Virol. 76, 3865–3872.

Yanagi, M., St-Claire, M., Emerson, S.U., Purcell, R.H., Bukh, J., 1999. In vivo analysis of the 3′ untranslated region of the hepatitis C virus after in vitro mutagenesis of an infectious cDNA clone. Proc. Natl. Acad. Sci. USA 96, 2291–2295.

Zhong, W., Uss, A.S., Ferrari, E., Lau, J.Y.N., Hong, Z., 2000. De novo initiation of RNA synthesis by hepatitis C virus nonstructural protein 5B polymerase. J. Virol. 74, 2017–2022.